

1 **TITLE**

2 **ASSAY FOR QUANTITATION OF HUMAN**
3 **DNA USING *Alu* ELEMENTS**

4 **BACKGROUND OF THE INVENTION**

5 **1. Field of the Invention**

6 [0001] The present invention concerns an assay for quantitating human DNA, particularly
7 quantitating human DNA present in samples containing extraneous nonhuman DNA.

8 **2. Description of the Related Art**

9 [0002] The quantitative detection of bio-materials in mixed forensic samples has been
10 approached using a variety of different systems. Early approaches to identify the origin of mixed
11 sample components involved the use of high-performance liquid chromatography (HPLC) based
12 methods. *See* H.F. Inoue *et al.*, *Species Identification of Blood and Bloodstains by High-*
13 *Performance Liquid Chromatography*, INT. J. LEGAL MED. 104:9-12 (1990). These methods
14 have proven useful, although the detection limits using these approaches are restrictive. The
15 detection of single copy nuclear DNA (Deoxyribonucleic acid) sequences has also been useful in
16 this regard, but is limited as a result of their single copy. Polymerase chain reaction (PCR) based
17 analysis of mitochondrial DNA sequences has also been used in the analysis of complex DNA
18 samples. The advantage of mitochondrial based DNA analyses derives from the fact that there
19 are many mitochondria per cell, and many mitochondrial DNA molecules within each

1 mitochondria making mitochondrial DNA a naturally amplified source of genetic variation. See
2 R.L. Cann *et al.*, *Mitochondrial DNA and Human Evolution*, NATURE 325:31-36 (1987).
3 However, a significant proportion of human forensic casework involves the analysis of nuclear
4 loci. See R. Chakrabarty *et al.*, *The Utility of Short Tandem Repeat Loci beyond Human*
5 *Identification: Implications for Development of New DNA Typing Systems*, ELECTROPHORESIS
6 20:1682-1696 (1999), making the identification and quantitation of human nuclear DNA a
7 paramount issue.

8 **[0003]** Commercially available products for human DNA quantitation include the Quantiblot®
9 (Applied Biosystems, Inc.) and the *AluQuant®* (Promega Corporation) systems. The
10 Quantiblot® system is based on the hybridization of a biotinylated oligonucleotide probe to
11 extracted DNA, followed by visual comparison of the colorimetric or chemiluminescent sample
12 results to the DNA standards. The *AluQuant®* system utilizes a luciferase reaction that results in
13 light output suitable for interpretation with a luminometer. M.N. Mandrekar *et al.*, *Development*
14 *of a Human DNA Quantitation System*, CROAT. MED. J. 42: 336-339 (2001).

15 **[0004]** These systems can become quite costly, particularly if a luminometer needs to be
16 purchased. The Quantiblot® and *AluQuant®* systems also detect non-human primate DNA, as
17 well as human DNA, and the detection limit for each of these systems is approximately 0.1 ng
18 (nanogram). These proprietary systems use technology that is not known to the public, because
19 it is apparently being maintained as a trade secret. Although Promega uses the trademark
20 “*AluQuant®*,” it is not known whether the technology actually uses *Alu* elements, and there is

1 considerable doubt in this regard because the system detects non-human DNA, which is not
2 consistent with the best *Alu*-based technology.

3 [0005] The use of *Alu* PCR amplification has been proposed as a more sensitive method for
4 the quantitation of genomic DNA compared to typical blot-based procedures currently used in
5 most forensic laboratories. See M.E. Sifis *et al.*, *A More Sensitive Method for the Quantitation*
6 *of Genomic DNA by Alu Amplification*, J. FORENSIC SCI. 47:589-592 (2002). Note that the term
7 “*AluQuant*” is a trademark of Promega Corporation and the *AluQuant®* system may not
8 necessarily be based on *Alu* mobile elements, per se.

9 [0006] *Alu* elements are transposable DNA elements which have amplified to a copy number
10 of over 1 million elements throughout primate evolution, thus producing a series of subfamilies
11 of *Alu* elements that appear to be of different genetic ages. The expansion of these elements
12 throughout primate evolution has created several recently integrated “young” *Alu* subfamilies
13 that are present in the human genome but are largely absent from non-human primates. M.A.
14 Batzer and P.L. Deininger, *Alu Repeats and Human Genomic Diversity*, NAT. REV. GENET.
15 3:370-379 (2002). These human-specific subfamilies only have a fraction of the copy number
16 compared to primate-specific elements, however, so that they are relatively less available for
17 assay use.

18 [0007] Recently Sifits, *et al.*, *supra*, reported a method whereby a fluorescently labeled
19 oligonucleotide PCR primer pair was designed to amplify a generic *Alu* sequence within primate
20 DNA. They reported that the assay had a sensitivity level of 100 - 2.5 pg of DNA, which is an
21 improvement over other assays with detection limits of 100-150 pg. However, their assay did

1 not distinguish human from non-human DNA in forensic samples that were contaminated with
2 non-human DNA, which frequently occurs.

3 [0008] Therefore, a need exists for a sensitive assay capable of quantitating human DNA
4 present in samples also containing extraneous non-human DNA.

5 SUMMARY OF THE INVENTION

6 [0009] Accordingly, it is an object of the present invention to provide a human DNA
7 quantitation method.

8 [0010] It is an object of the present invention to provide a sensitive assay for quantitating
9 human DNA present in samples also containing extraneous non-human DNA.

10 [0011] It is another object to provide a series of subfamily-specific *Alu*-based PCR assays.

11 [0012] In each case, the assay includes three basic steps. First, a DNA-containing sample to
12 be analyzed is provided. Second, amplification (preferably PCR (polymerase chain reaction)) of
13 predetermined genomic DNA sequences occurs. The sequences are located between adjacent
14 *Alu* elements, and the sequences are selected from *Alu* subfamilies present only in the human
15 genome. The second step results in an amplified DNA product. Third, the amplified DNA
16 product is compared with a reference.

17 [0013] For simply determining whether or not human DNA is present in a DNA sample, inter-
18 *Alu* and intra-*Alu* assays are described hereinbelow, including appropriate primers. Since the
19 loci selected for amplification are associated with *Alu* insertions present only in the human
20 genome (and absent from other primate or mammalian genomes), an amplified DNA product

1 occurs only for DNA samples containing human DNA. Thus, the reference for comparison
2 purposes is the original sample. The amplification of human template DNA in the process
3 described hereinbelow is an exponential increase, but there is essentially no amplification of non-
4 human DNA. The particular *Alu* subfamilies selected for purposes of the assay were the Yb8
5 and Yd6 subfamilies, because of their large characteristic diagnostic insertion or deletion. The kit
6 for performing this method includes the primers, polymerase chain reaction reagents such as
7 polymerase and buffers, and a reference for comparing the amplified multiple copies of the *Alu*
8 element to quantitate the human DNA. The kit optionally further includes reagents for extracting
9 and isolating DNA from the sample, reagents for detecting the human DNA on an agarose gel
10 stained with ethidium bromide, fluorescent dye and/or SYBR® green PCR core agents. The
11 primers used for the inter *Alu* PCR method include *Alu* 3- 5'GATCGCGCCACTGCACTCC 3'
12 (SEQ ID NO: 1) and *Alu* 5 - 5' GGATTACAGGCGTGAGCCAC 3' (SEQ ID NO: 2). The
13 primers used for the intra-Yb8-based PCR assay comprise 5'
14 CGAGGCGGGTGGATCATGAGGT 3' (SEQ ID NO: 3) and 5' TCTGTCGCCA
15 GGCCGGACT 3' (SEQ ID NO: 4). The primers used for the intra-Y6b-based PCR preferably
16 include 5' GAGATCGAGACCACGGTGAAA 3' (SEQ ID NO: 5) and 5'
17 TTTGAGACGGAGTCTCGTT 3' (SEQ ID NO: 6).

BRIEF DESCRIPTION OF DRAWINGS

[0014] A more complete appreciation of the invention, and many of the attendant advantages thereof, will be readily apparent as the same becomes better understood by reference to the

1 following detailed description when considered in conjunction with the accompanying drawings
2 in which like reference symbols indicate the same or similar components, wherein:

3 [0015] Figure 1 is a schematic diagram showing inter-*Alu* and intra-*Alu* PCR;

4 [0016] Figure 2 shows sequence alignment of *Alu* families;

5 [0017] Figures 3A through 3C show effective quantitation ranges of various *Alu* based assays;

6 [0018] Figures 4A through 4C show background amplification using non-human DNA
7 templates; and

8 [0019] Figure 5 illustrates human DNA detection on an agarose gel using an intra-Yd6 *Alu*
9 PCR assay.

10 **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

11 **Primer design and PCR amplification**

12 **Inter-*Alu* Primers**

13 [0020] Oligonucleotide primers selected for inter-*Alu* PCR are:

14 *Alu* 3- 5'GATCGCGCCACTGCACTCC 3' (SEQ ID NO: 1)

15 and

16 *Alu* 5 - 5' GGATTACAGGCGTGAGCCAC 3' (SEQ ID NO: 2).

17 **Intra-*Alu* Primers**

18 [0021] The *Alu* subfamily-specific intra-Yb8 primers selected are:

19 5' CGAGGCGGGTGGATGAGGT 3' (SEQ ID NO: 3) (position 48 to 69)

20 and

1 5' TCTGTCGCCCCAGGC*CGGACT* 3' (SEQ ID NO: 4) (position 273 to 254).

2 (The diagnostic bases are shown here in italics and underlined.)

3 [0022] The forward intra-Yd6 primer selected is:

4 5' GAGATCGAGACCAC/GGTGAAA 3' (SEQ ID NO: 5),

5 which crosses the characteristic Yd subfamily deletion, marked by the slash. The reverse intra-
6 Yd6 primer selected is:

7 5' TTTGAGACGGAGTCTCGTT 3' (SEQ ID NO: 6),

8 which contains a Yd6 subfamily-specific diagnostic mutation at the penultimate base.

9 [0023] Intra-*Alu* oligonucleotide primers are designed using either Primer3 software
10 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) or Primer Express
11 software (Applied Biosystems). The need to incorporate subfamily specific diagnostic mutations
12 into the primer design, as well as the high intrinsic GC content of *Alu* repeats, made it
13 challenging to identify oligonucleotide primers acceptable to the design software packages.
14 Oligonucleotides were purchased from Sigma-Genosys, Inc. The SYBR® green PCR core
15 reagent kit for quantitative PCR was purchased from Applied Biosystems, Inc.

16 **PCR Reaction Conditions**

17 [0024] PCR conditions were optimized for each assay with regard to annealing temperature
18 and concentrations of MgCl₂ and oligonucleotide primers. PCR reactions were carried out in 50
19 μl (microliter) using 1X SYBR green buffer, 1 mM dNTPs and 1.25 units AmpliTaq Gold®
20 DNA polymerase as recommended by the supplier. Inter-*Alu* PCR used 2 μM of each

1 oligonucleotide primer and 3mM MgCl₂. Each sample was subjected to an initial denaturation of
2 12 minutes at 95 °C to activate the AmpliTaq Gold®, followed by 40 amplification cycles of
3 denaturation at 95 °C for 20 seconds, 56 °C to anneal for 1 minute, and 1 minute of extension at
4 72 °C. Intra-Yb8 PCR used one µM of each oligonucleotide primer, 3 mM MgCl₂, an initial
5 denaturation of 12 minutes at 95 °C, followed by 40 amplification cycles of 95 °C for 15 seconds
6 and 74 °C for 1 minute to anneal and extend. Intra-Yd6 PCR used 0.5 µM of each oligonucleotide
7 primer, 5 mM MgCl₂, an initial denaturation of 12 minutes at 95 °C, followed by 40
8 amplification cycles of 95 °C for 15 seconds and 61 °C for 1 minute to anneal and extend. Each
9 reaction contained 49µl of PCR master mix and 1 µl of DNA template. Quantitative PCR
10 experiments were performed using an ABI Prism 7000 sequence detection system (Applied
11 Biosystems, Inc.) or a Bio-Rad i-cycler iQ real-time PCR detection system.

12 **Cell lines and DNA samples**

13 [0025] The cell lines used to isolate DNA samples were HeLa (*Homo sapiens*), pygmy
14 chimpanzee (*Pan paniscus*), and chimpanzee (*Pan troglodytes*) as described in M.A. Batzer, *et*
15 *al.*, *Standardized nomenclature for Alu repeats*, J. MOL. EVOL. 42:3-6 (1996). DNA from non-
16 primate species was obtained by tissue and blood extraction using the Wizard Genomic DNA
17 Purification kit (Promega) and samples provided by the Louisiana State University School of
18 Veterinary Medicine. Human control DNA (HeLa) was serially diluted 10-fold in 10 mM Tris
19 /0.1 mM EDTA (ethylenediaminetetraacetic acid) such that concentrations from 100 ng to 0.1 pg
20 were evaluated in replicates of two to four each.

1 **Data Analysis**

2 [0026] Data from the replicate DNA standards were exported from ABI Prism 7000 SDS
3 software® into a Microsoft® Excel spreadsheet where the mean value and standard deviation
4 were calculated for each point on the standard curve. The negative control (no template - NT)
5 was included in these calculations but was considerably lower than the last data point for inter-
6 *Alu* and intra-Yd6 standard curves and therefore does not appear on those charts, but does appear
7 on the intra-Yb8 chart. Using the Excel trendline option, a line of best fit was plotted with Y-
8 error bars equal to one standard deviation. Data from the non-human DNA cross-
9 hybridization/amplification experiments were exported to Excel in a similar manner and the
10 mean and standard deviation were calculated for each of four replicates. The Excel chart wizard
11 was used to construct bar graphs with Y-error bars equal to one standard deviation.

12 **Resulting Assays**

13 [0027] As a result of the foregoing techniques, several *Alu* element-based assays have been
14 developed for the rapid identification, and quantitation of human DNA present in a sample
15 mixed with non-human DNA. The assay approaches are respectively inter-*Alu* and intra-*Alu*
16 PCR based methods (*see* Figure 1) in conjunction with SYBR® green fluorescence detection
17 (trademark of Molecular Probes, Inc.).

18 [0028] Referring to Fig. 1, a schematic representation of inter-*Alu* and intra-*Alu* PCR, the
19 rectangles represent 5' – 3' or 3' – 5' orientation of *Alu* elements in the genome. During inter-*Alu*
20 PCR the 5' primer (unshaded arrows) and the 3' primer (small black arrows) amplify genomic

1 DNA sequences between adjacent *Alu* elements (dark lines) in any possible orientation, “tail-to-
2 tail” (T-T), “head-to-head” (H-H), or “tail-to-head” (T-H). During intra-*Alu* PCR, primers are
3 designed within the core body of the *Alu* element to amplify multiple copies of the element
4 derived from locations dispersed throughout the genome.

5 [0029] Inter-*Alu* PCR was originally developed, *see* D.L. Nelson *et al.*, *Alu polymerase chain*
6 *reaction: a method for rapid isolation of human-specific sequences from complex DNA sources*,
7 *PROC. NAT'L ACAD. SCI. USA* 86:6686-6690 (1989), to detect human DNA sequences in
8 somatic cell hybrids. Oligonucleotide primers were designed, based upon the primate *Alu*
9 consensus sequence and used to amplify unique human DNA sequences between adjacent *Alu*
10 repeats in an effort to isolate large regions of human DNA without the laborious task of first
11 creating a recombinant library from the somatic cell hybrids. Later, the technique was improved
12 by primers, *Alu* 5'/3' which amplified human DNA sequences between adjacent *Alu* repeats,
13 regardless of their orientation in the genome, and reportedly could generate PCR products
14 detectable by ethidium bromide staining from as little as 0.001 ng of human genomic DNA.
15 Inter-*Alu* PCR generates a complex pool of PCR amplicons of different sizes.

16 [0030] By contrast, intra-*Alu* PCR generates a homogeneous product composed entirely of
17 repeat core unit DNA sequences characteristic of the element being amplified. This approach is
18 similar in general concept to the primate *Alu* assay developed by Sifits *et al., supra*.

19 [0031] However, the assay of the present invention is instead based on the limited
20 amplification of members of those young *Alu* subfamilies that are present in the human genome
21 but are absent from non-human primate genomes. The subfamilies we selected for intra-*Alu*

1 evaluation were Yb8 and Yd6 because of the large diagnostic insertion or deletion that is
2 characteristic of these *Alu* families. The Yb8 subfamily consensus sequence contains eight
3 individual diagnostic mutations different from the ancestral *Alu*Y subfamily, as well as a seven
4 nucleotide insertion at position 253 (see Figure 2). Figure 2 illustrates sequence alignment of
5 *Alu* subfamilies. The consensus sequence (SEQ ID NO: 7) for the *Alu* Y subfamily is shown at
6 the top. The sequence (SEQ ID NO: 8) for *Alu* Yb8 subfamily is shown at the second line, and
7 the sequence (SEQ ID NO: 9) for Yd6 subfamily is shown at the last line. The dots represent the
8 same nucleotide as the *Alu* Y consensus sequence. Deletions are shown as dashes and mutations
9 are shown as the correct base for each subsequent subfamily. Sequences of the subfamily
10 specific intra-*Alu* oligonucleotide primers for amplification of the *Alu* core body sequence only
11 are shown in bold font.

12 [0032] There are estimated 1852 Yb8 *Alu* elements in the human genome. M.L. Carroll, *et al.*,
13 *Large-scale analysis of the Alu Ya5 and Yb8 subfamilies and their contribution to human*
14 *genomic diversity*, J. MOL. BIOL. 311:17-40 (2001) The recently reported Yd6 subfamily has six
15 subfamily specific diagnostic mutations as well as a twelve nucleotide deletion starting at
16 position 87 that defines the Yd lineage from the draft sequence of the human genome; and
17 estimated 97 Yd6 *Alu* subfamily members are present in the human genome. See M.A. Batzer *et*
18 *al.*, *Standardized nomenclature for Alu repeats*, J. MOL. EVOL. 42:3-6 (1996).

19 [0033] The following examples illustrate execution of the inventors' assays, using several
20 different test samples. Sample # 1 is a human DNA sample without non-human DNA present.
21 Sample # 2 is a human DNA sample with pygmy chimpanzee (*Pan paniscus*), and common

1 chimpanzee (*Pan troglodytes*) DNA also present. Sample # 3 is a human DNA sample with
2 other mammalian DNA also present (45% canine, 45% feline, and 10% human DNA).

3 **Example 1 - Inter-*Alu* Assay of Human DNA**

4 [0034] Sample #1 was prepared as follows. Approximately 1 μ l of capillary blood from a
5 male donor was washed twice in 500 μ l of distilled water and mixed with a conventional reaction
6 buffer for PCR. See, e.g., Chien *et al.*, J. BACTER. 127:1550 (1976). The mixture was mixed
7 with 100 μ l of a known amplification buffer – 10 mM Tris-HCl, pH 8.4 at 20 °C, 1.5 mM MgCl₂,
8 50 mM KCl, 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxycytidine 5'-triphosphate (dCTP),
9 2'-deoxyguanosine 5'-triphosphate (dGTP), 2'-deoxythymidine 5'-triphosphate (dTTP), 0.2 mM
10 each, 1.2 mM each primer. A conventional PCR followed, using approximately 40 cycles over
11 approximately 3 hours. The generation of PCR products or amplicons was directly monitored
12 using a PCR machine with an optical detection unit (quantitative PCR machine). The
13 amplification of human template DNA resulted in an exponential increase in the amount of
14 product produced.

15 **Example 2 - Inter-*Alu* Assay of Human/Primate DNA**

16 [0035] Sample #2 was prepared as follows. Equal amount of human and chimpanzee DNA
17 derived from tissue culture lines were mixed together and subjected to the same amplification
18 protocol outlined above. Using this approach we were able to compare the amplification derived
19 from a human template to that of a mixed template and that of a sample that contained only non-
20 human primate DNA (pygmy or common chimpanzee). The assay resulted in the exponential

1 amplification of DNA in samples that contained human DNA templates and essentially no
2 amplification in samples that only contained non-human primate DNA.

3 **Example 3 - Inter-*Alu* Assay of Human/Mammal DNA**

4 [0036] In this example DNA derived from cell lines or from peripheral lymphocytes derived
5 from a number of different mammals was subjected to inter-*Alu* PCR and optical detection.
6 Since *Alu* repeats are contained only within members of the primate order and recently integrated
7 *Alu* elements are found only within the human genome, little or no amplification was to be
8 expected in the test samples that contained the DNA of other animals as templates. As was to be
9 expected, essentially no amplification of PCR products was detected in these samples. That
10 demonstrated the specificity of the assay.

11 **Example 4 - Intra-*Alu* Yb8 Assay of Human DNA**

12 [0037] Sample # 1 of Example 1 was subjected to the procedure of Example 1, using Intra-*Alu*
13 Yb8 based PCR in place of the Inter-*Alu* material used in Example 1. Under this approach, it
14 was to be anticipated that the generation of a PCR product would occur only from samples that
15 contained human DNA and that no PCR products would be generated from the samples that
16 contained other DNA sources. The reason is that the recently integrated *Alu* elements are
17 restricted to the human genome. The anticipated result was the result of the assay.

18 **Example 5 - Intra-*Alu* Yb8 Assay of Human/Primate DNA**

19 [0038] Sample # 2 of Example 2 was subjected to the procedure of Example 4, using Intra-*Alu*
20 Yb8 amplification in place of the Inter-*Alu* based PCR amplification used in Example 2. In this
21 assay, templates derived from human DNA, templates with only chimpanzee DNA (a non-human

1 primate), and templates that were mixed and contained human and non-human primate DNA
2 were subjected to amplification. Using these templates, only samples that contained human
3 DNA generated PCR products. Samples that contained only non-human primate DNA did not
4 amplify, thereby indicating the specificity of the assay.

5 **Example 6 - Intra-*Alu* Yb8 Assay of Human/Mammal DNA**

6 [0039] Sample # 3 of Example 3 was subjected to the procedure of Example 4, using Intra-*Alu*
7 Yb8 PCR in place of the Inter-*Alu* PCR. In this approach DNA from a human and several other
8 mammals (e.g. cow, sheep, pig) were subjected to amplification either individually or mixed
9 together. Only samples that contained human DNA generated PCR products in the assay.
10 Samples that contained only the DNA of non-human mammals (cow, sheep, pig *etc.*) did not
11 amplify in the assay, thereby demonstrating that the assay is specific for human DNA.

12 **Example 7 - Intra-*Alu* Yd6 Assay of Human DNA**

13 [0040] Sample # 1 of Example 1 was subjected to the procedure of Example 1, using Intra-*Alu*
14 Yd6 amplification in place of the Inter-*Alu* based PCR amplification used in Example 2. In this
15 assay, templates derived from human DNA, templates with only chimpanzee DNA (a non-human
16 primate), and templates that were mixed and contained human and non-human primate DNA
17 were subjected to amplification. Using these templates, only samples that contained human
18 DNA generated PCR products. Samples that contained only non-human primate DNA did not
19 amplify, thereby indicating the specificity of the assay.

20 **Example 8 - Intra-*Alu* Yd6 Assay of Human/Mammal DNA**

1 [0041] Sample # 2 of Example 2 was subjected to the procedure of Example 2, using Intra-*Alu*
2 Yd6 PCR in place of the Inter-*Alu* PCR. In this approach, DNA from a human and several other
3 mammals (e.g. cow, sheep, pig) were subjected to amplification either individually or mixed
4 together. Only samples that contained human DNA generated PCR products in the assay.
5 Samples that contained only the DNA of non-human mammals (cow, sheep, pig etc.) did not
6 amplify in the assay, thereby demonstrating that the assay is specific for human DNA.

7 **Example 9 - Intra-*Alu* Yd6 Assay of Human/Animal DNA**

8 [0042] Sample # 3 of Example 3 was subjected to the procedure of Example 3, using Intra-*Alu*
9 Yd6 PCR in place of the Inter-*Alu* PCR. In this approach, DNA from a human and several other
10 mammals (e.g. cow, sheep, pig, chicken) were subjected to amplification either individually or
11 mixed together. Only samples that contained human DNA generated PCR products in the assay.
12 Samples that contained only the DNA of non-human mammals (cow, sheep, pig, chicken etc.)
13 did not amplify in the assay, thereby demonstrating that the assay is specific for human DNA.

14 [0043] Figure 3 shows the effective quantitation ranges of various *Alu* PCR based assays using
15 SYBR green fluorescence detection. Figure 3A depicts the effective range for Inter-*Alu* PCR.
16 Figure 3B depicts the effective range for Intra-*Alu* Yb8. PCR Figure 3C depicts the effective
17 range for Intra-*Alu* Yd6 PCR. The PCR cycle at which the fluorescent signal crosses baseline is
18 considered to be the threshold cycle, plotted on the Y-axis. The fluorescent signal produced by a

1 10-fold dilution series of human DNA is plotted as the mean of 2-4 replicates, +/- one standard
2 deviation. The R² value is at least 99% for all three standard curves.

3 [0044] Referring now to Figure 4, showing background amplification using non-human DNA
4 templates, it is seen that substantially greater cross-amplification exists for inter-*Alu* PCR based
5 assays using SYBR green fluorescence detection relative to the subfamily-specific intra-*Alu*
6 assays. Figure 4 shows the cross amplification of non-human template DNA for inter-*Alu*
7 (Figure 4A); intra-*Alu* Yb8 (Figure 4B); and intra-*Alu* Yd6 (Figure 4C). The PCR cycle at which
8 the fluorescent signal crosses baseline is considered to be the threshold cycle, plotted on the Y-
9 axis (mean of three replicates, +/- one standard deviation).

10 [0045] The inter-*Alu* assay detected human, pygmy chimpanzee (*Pan paniscus*), and common
11 chimpanzee (*Pan troglodytes*) DNA (2 ng per reaction), but also produced some background
12 amplification using DNA from other mammals as template. The intra-*Alu* Yb8 assay (2 ng DNA
13 per reaction) and the intra-*Alu* Yd6 assay (10 ng DNA per reaction) are entirely human-specific
14 with essentially no background amplification using pygmy chimpanzee (the non-human primate
15 thought to be the closest genetic relative to humans) DNA as a template after 30 cycles of PCR.

16 [0046] The inter-*Alu* based PCR assays had a linear quantitation range of 10 ng to 0.001 ng as
17 shown by the standard curve (Figure 3A). The mean value of the negative control was 31.3 with
18 a 0.4 standard deviation. This assay was expected to amplify DNA from non-human primates
19 (*Pan paniscus* and *Pan troglodytes*) as well as human DNA. However significant background
20 amplification was also detected in other species when tested with an equivalent amount (2 ng) of
21 non-human DNA (see Figure 4A). This limits the effective minimum threshold quantitation

1 level of this inter-*Alu* PCR based assay to 0.01 ng (threshold PCR cycle 22) rather than 0.001 ng,
2 and restricts the range of the inter-*Alu* based PCR assay to 1000-fold when testing DNA samples
3 from complex sources. To further evaluate the potential background amplification that non-
4 human DNA might have on the assay with respect to human DNA quantitation, an artificial
5 “domestic DNA mix” (10 ng/ml) was prepared that contained 45% canine, 45% feline, and 10%
6 human DNA. The results of that experiment (mean of duplicates, +/- one standard deviation)
7 are shown as data point “a” in Figure 3A. Since 10% of 10 ng is 1 ng, the assay was able to
8 accurately quantitate the human DNA in the mixed sample.

9 [0047] The intra-Yb8 based PCR assay had a low-scale linear quantitation range of 10 ng to
10 0.001 ng (10,000-fold) as shown by the standard curve (*see* Figure 3 B). The value of the no
11 template (NT) negative control was not significantly different than the last data point (0.0001 ng).
12 Background amplification was detected in *Pan paniscus* (pygmy chimpanzee), the most closely
13 related non-human primate, following 31 cycles of PCR and was detected in other species in
14 trace amounts following 36 cycles of PCR using an equivalent amount of DNA template (2ng)
15 (*see* Figure 4B). This demonstrates that the intra-Yb8 assay is human specific to 0.01ng, and
16 specific to only human and pygmy chimpanzee to 0.001ng (1 pg) when evaluating mixed DNA
17 samples. If a sample is known to consist of only human DNA, the detection limit of this assay
18 may extend between 1 pg and 0.1 pg but must accompany an appropriate no template control.

19 [0048] The intra-Yd6 based PCR assay had a high-scale linear quantitation range of 100 ng to
20 0.1 ng (1000- fold) as shown by the standard curve (*see* Figure 3C). The mean value of the
21 negative control was 35.7 with a 1.17 standard deviation. No signal was detected from any of

1 the non-human species tested, making this assay absolutely human specific within its
2 quantitation range (*see* Figure 4C). The intra-Yd6 assay not only extends the linear quantitative
3 range of the intra-*Alu* assays combined, it also allows for human DNA detection and rough
4 quantitative estimates to be performed by simple, inexpensive agarose gel electrophoresis as an
5 initial screening tool (*see* Figure 5). The quantitation of the human DNA in the mixture sample
6 can be achieved by comparing the intensity of the signal on an agarose gel stained with ethidium
7 bromide from unknown samples with the intensity of standard human DNA or from the
8 calibration curve, which can be generated from the results for the standard human DNA samples.

9 [0049] Figure 5 illustrates human DNA detection on an agarose gel using the intra-Yd6 *Alu*
10 PCR assay. Following 30 cycles of conventional PCR using the intra-Yd6 oligonucleotide
11 primers, amplicons were chromatographed on a 2% agarose gel stained with ethidium bromide.
12 Using the human DNA standards on the left, samples A through C were positive for the presence
13 of human DNA (*i.e.*, they generated a PCR amplicon) while samples D and E were not positive
14 (10 ng chimpanzee or rat DNA template, respectively). Samples A-C contain 30, 5, and 0.5 ng
15 of human DNA, respectively. These values are reasonably consistent with expected values based
16 on empirical observations of fragment intensity. This demonstrates that the assay is a simple,
17 rapid, and inexpensive means of human DNA detection that also provides quantitative estimates
18 of human template DNA.

19 **Summary of Results**

1 **[0050]** The vast majority of human forensic casework involves the analysis of nuclear loci
2 making the quantitation of human nuclear DNA a paramount issue. There are several advantages
3 to the *Alu*-based methods disclosed herein for the rapid identification and quantitation of human
4 DNA over commercially available systems and other recently reported methods. First, these
5 assays can use a polymerase chain reaction. If an ample amount of DNA is available for testing,
6 human-specific DNA detection and quantitative estimates can be performed by simple agarose
7 gel analysis using the intra-Yd6 *Alu* based PCR assay as an initial screening tool. The addition
8 of SYBR green PCR core reagents to the amplification protocol allows accurate quantitation
9 using any qPCR system. No additional special expertise or unique (or expensive) equipment,
10 such as a luminometer or automated DNA sequencer/genotyper is required. This format alone
11 minimizes the cost of performing these analyses on a large-scale and gives most forensic
12 laboratories with average resources the ability to perform these assays.

13 **[0051]** The inventors have also systematically tested each assay for human specificity,
14 especially with regard to closely related non-human primates and in multiple diverse human
15 genomes. In contrast, documentation associated with other currently available methods is vague
16 with respect to the cross-hybridization/amplification of other closely related species. In addition,
17 the range of quantitation using the combined intra-*Alu* based assays (Yb8 and Yd6 subfamilies)
18 is approximately 10^5 based on the above described 10-fold dilution series experiments. By
19 contrast, the current commercial quantitation systems such as *AluQuant*® and *Quantiblot*® have
20 a 500-fold and 100-fold quantitation range, respectively. In other words, the low range detection
21 limit of the intra-Yb8 assay described here exceeds the commercial systems by a minimum of

1 100-fold and it also exceeds the method recently reported by Sifis *et al.*, *supra*, by at least 2.5-
2 fold. Further, since Sifis *et al.*, *supra*, do not address possible mammalian cross-amplification
3 with their assay, the intra-Yb8 method reported here is even more sensitive for the identification
4 of human DNA from complex sources.

5 [0052] The high copy number of *Alu* repeats in the human genome makes these assays ideal
6 for human DNA detection and quantitation. When inter-*Alu* PCR was first developed over 15
7 years ago, it was revolutionary in allowing sensitive amplification and detection of human DNA
8 from somatic cell hybrids while circumventing the laborious task of first creating a recombinant
9 library. The detection limit reported then of 0.001 ng of human DNA is consistent with the
10 findings described hereinabove. However, the fact that the present inter-*Alu* PCR method
11 amplifies unique genomic DNA sequences between adjacent *Alu* repeats, creating a complex
12 pool of various sized PCR amplicons, makes that method vulnerable to greater cross-
13 amplification potential with DNA from other mammals compared to the intra-*Alu* methods of the
14 instant invention. Furthermore, the inter-*Alu* PCR method requires the use of fluorescence or
15 radioactive isotope in the PCR reaction to be quantitative, whereas the intra-*Alu* methods are
16 more amenable to additional detection schemes such as ethidium bromide or TaqMan®
17 chemistry.

18 [0053] In practicing the instant invention, several caveats are in order. The inventors have
19 observed that the high T_m of the intra-Yb8 *Alu* based primers is essential to the elimination of
20 artifact amplicons from DNA of other species as a result of sequence similarity to SINE elements
21 from other species. This was manifested when the inventors attempted to design a similar intra-

1 *Alu* PCR assay using the Ya5 subfamily consensus sequence, but were unable eliminate
2 background amplification. Optimization of oligonucleotide primer concentration also proved to
3 be an important component in the development of these *Alu* based PCR assays. Compared to
4 single copy PCR reactions, the high number of target sequences in these assays made it
5 important to have sufficient amounts of primer without compromising PCR amplification
6 efficiency.

Kits

[0054] It is considered that the scope of the invention extends to kits used to practice the assays of the invention. Thus, it is contemplated that the invention would be exploited by marketing kits for DNA quantitation of unknown biological samples, using the principles and procedures described hereinabove. For example, a DNA quantitation kit comprises a reagent mix and a DNA control. The control contains a predetermined amount of human DNA suspended in an appropriate salt solution. The reagent mix, often termed as Primer Mix, contains the primers, salts, and other chemicals such as dNTPs, in proportions suitable to obtain the desired results. The following examples illustrate representative kits for practicing embodiments of the invention.

Example 10- Kit for Quantitating DNA

[0055] A kit suitable for performing quantitating DNA includes the primers and polymerase and buffers for the PCR reaction. If the quantitation of DNA by using the above procedure is

1 used at a crime scene, the kit can optionally include the reagents for extracting and isolating
2 DNA from evidence.

3 [0056] For example, the kit includes PCR tubes, sterile water, sterile TLE, SYBR® Green
4 core reagent kit and Human DNA controls, and a pair of primers (e.g., (1) *Alu* 3-
5 5'GATCGCGCCACTGCACTCC 3' (SEQ ID NO: 1) and *Alu* 5 - 5'
6 GGATTACAGGCGTGAGCCAC 3' (SEQ ID NO: 2), (2) 5'
7 CGAGGCGGGTGGATCATGAGGT 3' (SEQ ID NO: 3) (position 48 to 69) and 5'
8 TCTGTCGCCAGGCCGGACT 3' (SEQ ID NO: 4) (position 273 to 254), or (3) 5'
9 GAGATCGAGACCACGGTGAAA 3' (SEQ ID NO: 5) and 5' TTTGAGACGGAGTCTCGTT
10 3'(SEQ ID NO: 6)).

11 [0057] The concentrations of each reagent can be properly selected depending on the intended
12 use of the kit.

13 [0058] The following examples illustrate the use of the foregoing kits to perform assays in
14 accordance with the invention.

15 **Example 11 - Procedure for Intro-*Alu* Yb8 Assay**

16 [0059] Human DNA standards (HeLa or other known control) were prepared in steril TLE to
17 10 ng/µl and serially diluted 10-fold with TLE. Unknown DNA samples in TLE were prepared
18 as was done with the standards. As with any quantitative assay, optimal quantitation occurs in
19 the middle of the linear range of the standard curve. Therefore, it may be useful to analyze more

1 than one concentration of an unknown DNA sample. Stock primers were reconstituted in sterile
2 TLE to a concentration of 100 μ M. Then, 0.5ml of working solution of each primer at 10 μ M
3 was made by diluting 50 μ l of each stock with 450 μ l of TLE. This represents a 10X working
4 concentration of each primer for the intra-*Alu* Yb8 human DNA quantitative PCR assay. PCR
5 tubes, strips or plate as needed were prepared, and a template showing the location of the
6 negative control (TLE), the positive controls (A 10-fold serial dilution of human control DNA
7 from 10 ng/ μ l), and each unknown DNA sample to be quantified was made. 1 μ l of DNA
8 template was pipetted into each appropriate well. Then a master mix of all remaining PCR
9 reagents as shown in Table 1 was prepared. 49 μ l of master mix was pipetted into each well to
10 make a final PCR reaction volume of 50 μ l, as recommended by the manufacturer (SYBR®
11 Green PCR core reagent kit). PCR tubes or plate is placed into the Z-PCR machine. The PCR
12 was performed by subjecting to an initial denaturation of 12 minutes at 95 °C, followed by 40
13 amplification cycles of 95 °C for 15 seconds and 61 °C for 1 minute to anneal and extend. The
14 amplification experiments were performed using an ABI prism 7,000 sequence detection system
15 (Applied Biosystems, Inc.) or a Bio-Rad i-cycler iQ real- time PCR detection system. A
16 calibration curve was generated from the results for the standard DNA samples. The quantity of
17 the human DNA in the unknown sample was computed from the calibration curve.

1 TABLE 1

2	OPCR reagents	Final conc.	volume (μ l)	example for 32
3	Sterile Water		23.75	760
4	SYBR® Green PCR Buffer (10X) *	1X	5	160
5	Intra-Alu Yb8-Forward (10 μ M; 10X)	1 μ M	5	160
6	Intra-Alu Yb8-Reverse (10 μ M; 10X)	1 μ M	5	160
7	dNTPs (12.5 mM) *	1 mM	4	128
8	MgCl ₂ (25 mM) *	3 mM	6	192
9	AmpliTaq Gold (5 Unit/ μ l) *	1.25 Units	0.25	8
10	Total : (49 μl X 32)		49 μl	1568

11 * Provided in the SYVR green PCR reagent core kit (ABI part number 4304886)

12 [0060] Several alternative methods for human DNA detection, and quantitation, inter-*Alu* and
 13 intra-*Alu* based PCR, have been described above. Although the original inter-*Alu* PCR method
 14 revolutionized human DNA detection when it was first developed, the instant disclosure has now
 15 demonstrated that the intra-*Alu* based PCR method is more suited to modern forensic needs. The
 16 subfamily-specific intra-*Alu* assays presented herein, Yb8 and Yd6, employ the amplification of
 17 a high copy number of target sequences to achieve very sensitive human-specific DNA detection,
 18 while simultaneously maintaining some of the same advantages of a single locus PCR. For
 19 example, the amplified products are a uniform size amplicon in each assay, conducive to easy

1 visualization. These amplicons are also short enough (226 bp for Yb8 and 200 bp for Yd6) to
2 tolerate sheered or degraded DNA often associated with forensic applications.

3 [0061] While the invention has been described in connection with specific and preferred
4 embodiments thereof, it is capable of further modifications without departing from the spirit and
5 scope of the invention. This application is intended to cover all variations, uses, or adaptations
6 of the invention, following, in general, the principles of the invention and including such
7 departures from the present disclosure as come within known or customary practice within the
8 art to which the invention pertains, or as are obvious to persons skilled in the art, at the time the
9 departure is made. It should be appreciated that the scope of this invention is not limited to the
10 detailed description of the invention hereinabove, which is intended merely to be illustrative, but
11 rather comprehends the subject matter defined by the following claims.